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Role of the gradient slope during the product internal recycling for the multicolumn countercurrent solvent gradient purification of PEGylated proteins

Tae Keun Kim[a], Abdallah Ayub Bham[a], Ismaele Fioretti[a], James Angelo[b], Xuankuo Xu[b], Sanchayita Ghose[b], Massimo Morbidelli[a], Mattia Sponchioni[a,*]

[a] *Department of Chemistry, Materials and Chemical Engineering, Politecnico di Milano, Via Mancinelli 7 20131 Milano, Italy*
[b] *Biologics Process Development, Global Product Development and Supply, Bristol Myers Squibb, Inc., Devens, MA, 01434, USA*

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ABSTRACT

Protein PEGylation, i.e. functionalization with poly(ethylene glycol) chains, has been demonstrated an efficient way to improve the therapeutic index of these biopharmaceuticals. We demonstrated that Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is an efficient process for the separation of PEGylated proteins (Kim et al., Ind. and Eng. Chem. Res. 2021, 60, 29, 10764–10776), thanks to the internal recycling of product-containing side fractions. This recycling phase plays a critical role in the economy of MCSGP as it avoids wasting valuable product, but at the same time impacts its productivity extending the overall process duration. In this study, our aim is to elucidate the role of the gradient slope within this recycling stage on the yield and productivity of MCSGP for two case-studies: PEGylated lysozyme and an industrially relevant PEGylated protein. While all the examples of MCSGP in the literature refer to a single gradient slope in the elution phase, for the first time we systematically investigate three different gradient configurations: i) a single gradient slope throughout the entire elution, ii) recycling with an increased gradient slope, to shed light on the competition between volume of the recycled fraction and required inline dilution and iii) an isocratic elution during the recycling phase. The dual gradient elution proved to be a valuable solution for boosting the recovery of high-value products, with the potential for alleviating the pressure on the upstream processing.

1. Introduction

A critical issue that is hampering the accessibility to conjugated proteins is the availability of selective and efficient purification processes allowing for the isolation of the desired molecule from conjugation by-products, as these most often share very similar physicochemical properties with the target [1–6]. This is the case of PEGylated proteins, for which the mono-PEGylated species need to be separated from multi-PEGylated or non-PEGylated impurities [1,7–9]. To achieve the required purity target, the dominant technique is single-column cation exchange chromatography, exploiting the alteration of the protein net charge introduced by PEGylation. Indeed, multi-PEGylated species are characterized by a lower charge and are weakly bound impurities (W) compared to the mono-PEGylated protein (product, P), while the least neutralization for the native protein makes it a strongly bound impurity (S) [7,10,11]. The product hence elutes in the middle of these two classes of impurities and partially overlaps with their elution peak.

A critical issue of single-column chromatography for these difficult center-cut purifications is the intrinsic tradeoff between purity and yield [5,9,12–14]. We demonstrated that for many molecules, including PEGylated proteins, this tradeoff could be alleviated through Multicolumn Countercurrent Solvent Gradient Purification (MCSGP), due to the internal recycling of product-containing side fractions [5,10,13–33]. This process, based on two twin columns, is schematically depicted in Fig. 1**A**. For the detailed description of the characteristic steps, the reader is addressed to [14].

Considering a linear gradient elution as in Fig. 1**B**, it is known that in a single-column batch chromatography the shallower the gradient, the higher the resolution of the separation, and hence, for a given purity specification, the larger the collection window (*i.e.* t_3-t_4 interval in Fig. 1**B**). This ensures higher yields but at the expenses of lower productivity. In the opposite direction, the steeper the gradient, the poorer the resolution and hence the smaller the

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* Corresponding author.
E-mail address: mattia.sponchioni@polimi.it (M. Sponchioni).

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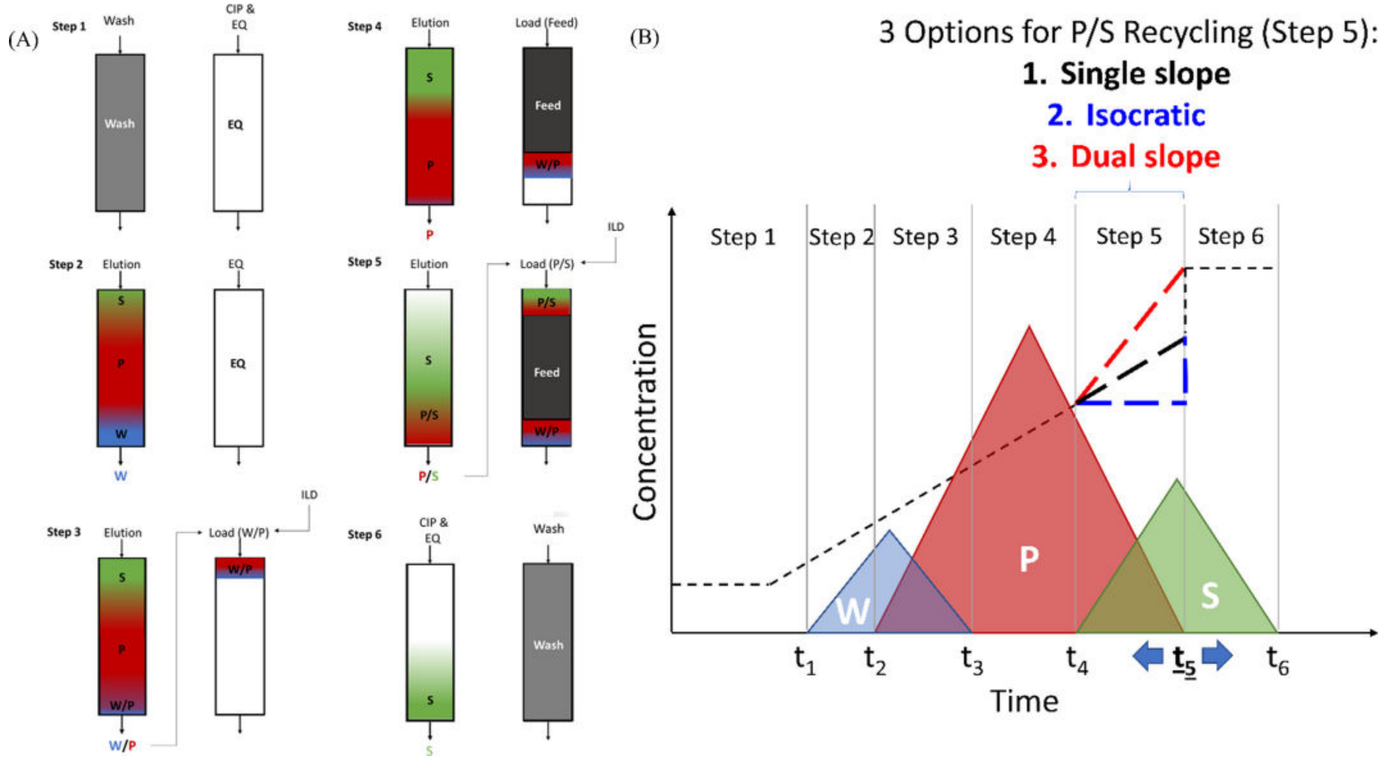

Fig. 1. (A) Schematic of the two-column MCSGP process with its characteristic 6 steps. Reproduced with permission from [18]. Copyrights Elsevier 2022. (B) Typical chromatogram of a center-cut purification with the product (P) partially co-eluting with weak (W) and strong (S) impurities under a linear gradient (**—**) elution. The three possible gradient slopes investigated in this study for the interval t_4 – t_5 are shown: i) same slope as before t_4 (dashed bold black line), ii) higher gradient slope (dashed red line), iii) isocratic elution (dashed blue line). The specific gradient slope in this phase affects the timepoint t_5 as well as the process yield and productivity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

collection window at acceptable purity, which compromises the yield but potentially improves the productivity. A smart solution to avoid this tradeoff is then running the elution phase until t_4 with a mild gradient slope in order to improve the yield, while increasing it from t_4 to t_5 in order to save the productivity. For MCSGP, reaching the same conclusion is not trivial. In fact, the time interval t_4 – t_5 is characterized by the recycling of the P/S overlap from the upstream to the downstream column. This needs to be accompanied by an in-line dilution (ILD) in order to lower the modifier concentration of this recycled fraction and make it to re-adsorb on the receiving column. Therefore, increasing the gradient slope in the interval t_4 – t_5 would decrease the volume of the P/S overlap to be recycled, but it would require a higher dilution factor because of the higher modifier concentration. This ILD requires the reduction of the flow rate of the recycled fraction to ensure constant residence time in the receiving column, thus penalizing the productivity. Therefore, we postulated that this tradeoff between yield and productivity introduced by the ILD can be manipulated by acting on the gradient slope in the interval t_4 – t_5.

To shed light on the role of the ILD and verify this hypothesis, in this work the performances of MCSGP were systematically investigated in the case of three elution modes for the P/S overlap between t_4 and t_5, as shown in Fig. 1**B**: i) linear gradient with the same slope as between t_1 and t_4 (dashed bold black line in Fig. 1**B**), which is the typical configuration; ii) second gradient with higher slope than the one between t_1 and t_4 (dashed red line in Fig. 1**B**); iii) isocratic with the same modifier concentration reached in t_4 (dashed blue line in Fig. 1**B**). All the operating parameters are instead fixed and equal for these three processes until t_4. In this analysis, the key process parameter is t_5, affected by the gradient slope and the dilution factor in the interval t_4 – t_5. In fact, the steeper the gradient, the lower the P/S volume to be recycled but, at the same time, the higher the dilution factor, requiring a more accentuated decrease in the recycling flow rate to allow mixing with the dilution buffer. As one could expect, the isocratic elution with the shallowest elution gradient would require the lowest dilution factor for the P/S recycling while its volume would be the largest; for the dual slope elution, the opposite trend would be observed with the highest dilution factor and the smallest volume. These two effects have an opposite outcome on the productivity and it is difficult to predict *a priori* the prevailing one, which might be dependent on the molecule to be purified. For this reason, the systematic analysis of the role of the gradient slope in the P/S recycling phase was conducted for two PEGylated proteins. PEGylated lysozyme was investigated first, as it is a well-known protein standard for cation exchange chromatography [9,34–39]. Then, the analysis was performed on an industrially relevant PEGylated protein (hereinafter PEG-target) characterized by loss of some product in the strip phase. As the main process parameters for MCSGP of these two case studies had been previously optimized [5,18], we leveraged this previous work for designing the process until t_4 and changed the gradient slope in the interval t_4-t_5 according to the configuration under investigation.

We found that a dual gradient slope is useful for improving the recovery of the product even if at the expenses of reduced productivity. Therefore, this dual slope presented herein is a valuable solution when purifying high-value drugs. In fact, the higher yield can alleviate not only the upstream processing but also any subsequent downstream labor required to match a targeted amount of drug to be processed. Therefore, although a temporary loss in productivity is presented, this yield-gaining dual slope elution by MCSGP can help whenever high product recovery is extremely critical.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) methyl ether (mPEG, MW = 5000, Sigma Aldrich), Succinic anhydride (SA, ≥99%, MW = 100.07, Sigma Aldrich), 4-Dimethilamino pyridine (DMAP, ≥99%, MW = 122.17, Sigma Aldrich), Anhydrous dichloromethane (DCM, ≥99%, MW = 84.93, Sigma Aldrich), Diethyl ether (≥99%, MW = 74.12, Sigma Aldrich), N−Hydroxysuccinimide (NHS, 98%, MW = 115.09, Sigma Aldrich), N,N'-Diciclohexylcarbodiimide (DCC, ≥99%, MW = 206.33, Sigma Aldrich), Deuterated chloroform (Chloroform-d, 99.8%, MW = 120.38, Sigma Aldrich), Lysozyme from chicken egg white (Lys, ≥99%, MW = 14,300, Sigma Aldrich), Sodium phosphate monobasic (NaH_2PO_4, ≥99%, MW = 119.98, Sigma Aldrich), Sodium phosphate dibasic ($Na_2H_2PO_4$, ≥99%, MW = 141.96, Sigma Aldrich), 2-(N-morpholino) ethanesulphonic acid hydrate (MES, ≥99%, MW = 213.25, Sigma Aldrich), Sodium hydroxide (NaOH, ≥99%, MW = 40.00, Sigma Aldrich), Sodium chloride (NaCl, ≥99%, MW = 58.44, Sigma Aldrich), hydrochloric acid 37%(HCl, ≥99%, MW = 36.46, Sigma Aldrich), Acetic acid (≥99%, MW = 60.05, Sigma Aldrich), potassium phosphate monobasic (KPi monobasic, 99%, 136.09, Sigma-Aldrich), Trizma base (Tris, ≥99.9%, MW = 121.14, Sigma-Aldrich), Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl, ≥99.0%, MW = 157.60, Acros Organics), Ethanol (EtOH, MW = 46.07, Sigma-Aldrich), MiniChrom column Eshmino CPX (CPX column, 8 × 100 mm, 50 μm, Merck), ProPac WCX-10 (WCX column, 4 × 250 mm, 10 µm, Thermo Scientific), HiScreen Q FF (Q FF column, 7.7 × 100 mm, 90 μm, Cytiva), TSKgel G3000SWXL size exclusion column (SEC column, 7.8 × 300 mm, 5 μm, 250 Å, Tosoh Bioscience), deionized water (DW) were used for experimentations.

All buffers were filtered with 0.45 μm polyvinylidene fluoride filters (PVDF, Merck Millipore) and the buffers used for HPLC were also degassed for chromatographic purposes.

The amine conjugation method was employed for PEGylation of lysozyme. This three-step method consists of i) conversion of mPEG to mPEG-carboxylic acid, ii) activation of the carboxylate group to succinimidyl ester, thereby synthesizing mPEG-NHS, and iii) conjugation of mPEG-NHS to lysozyme. The detailed description of the synthetic protocol can be found in a previous work [5]. The final concentration and pH of mPEG-lysozyme were 5.0 g L^{-1} and 7.0 ± 0.2, respectively. Each process intermediate was dissolved in 1 mL of deuterated chloroform and analyzed using proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The final product, mPEG-lysozyme, was analyzed by WCX-HPLC for characterizing its composition.

The industrially relevant PEGylated protein was provided by Bristol Myers Squibb at an initial concentration of 35.5 g L^{-1}. The protein was diluted using a dilution buffer (50 mM Tris, pH 8.8) to reach the final pH, conductivity, and concentration of 8.1 ± 0.3, ≤ 1.7 mS cm^{-1}, and 1.25 ± 0.25 g L^{-1}, respectively [18]. This mixture was then characterized using SE-HPLC.

2.2. Analytical chromatography

For the analysis of the feed and the fractions collected in PEG-lysozyme purification, a weak cation exchange high performance liquid chromatography (WCX-HPLC) using a ProPac WCX-10 column (4 × 250 mm) was employed. WCX A buffer (20 mM MES, pH 5.5) and WCX B buffer (20 mM MES, 500 mM NaCl, pH 5.5) were prepared by filtration and degassing. The analysis was performed on an Agilent 1100 series HPLC at 1.0 mL min^{-1} at room temperature with 20 μL injection. The analytical method is summarized in **Table S1**. The UV absorbance was recorded at 280 nm and the purity of each fraction was calculated based on the areas of the peaks of the different species. In particular, using the area of PEG-lysozyme peak ($Area_{target}$) and the total area of the fraction ($Area_{total}$), the purity of each fraction was computed according to Eq. (1).

$$Purity = \frac{Area_{target}}{Area_{total}} \times 100\% \tag{1}$$

The yield of PEG-lysozyme of a product pool was computed by the ratio of the mass of PEG-lysozyme in that product pool ($m_{recovered}$) and the total mass injected into the system (m_{total}), as in Eq. (2).

$$Yield\ (\%) = \frac{m_{recovered}}{m_{total}} \times 100\% \tag{2}$$

By summing up the time for each phase, including equilibration (t_{eq}), load feed (t_{Load}), wash (t_{Wash}), elution (t_{elu}), strip (t_{strip}), CIP (t_{CIP}), and storage (t_{Sto}), the total time of a batch purification, or t_{total}, is calculated as shown in Eq. (3). With all parameters obtained, productivity could be computed using Eq. (4).

$$t_{total} = t_{EQ} + t_{Load} + t_{Wash} + t_{Elu} + t_{Strip} + t_{CIP} + t_{Sto} \tag{3}$$

$$Productivity = \frac{m_{total} \times Yield}{CV \times t_{total}} = \frac{m_{recovered}}{CV \times t_{total}} \tag{4}$$

Using a TSKgel G3000SWXL size exclusion column (7.8 × 300 mm) on the HPLC, the feed and fractions obtained by the purification of the industrially relevant PEGylated protein were analyzed. Following 10 μL injection of an analyte, SEC A buffer (95% Phosphate-buffered saline (PBS) + 5% EtOH, pH 7.2 −7.4) was used for the elution over 30 min at 0.8 mL min^{-1} at 25 °C. To store the column, SEC B buffer (20% EtOH) was loaded for 120 min at 0.4 mL min^{-1} at the same temperature. The purity, yield and productivity were computed according to Eqs. (1)–(4).

2.3. Single-column batch purification

The preparative chromatographic purification of mono-PEGylated lysozyme from PEGylation impurities was performed on a Contichrom CUBE 30 using an Eshmuno CPX resin (8 × 100 mm, column volume (CV)=5.02 mL, particle size 50 μm). The process was conducted at a constant velocity of 300 cm hr^{-1}, and thus the residence time (RT) was 2 min.

In order to highlight the impact of the gradient slope in the interval t_4 - t_5 on batch and MCSGP performances, we compared the isocratic elution, prosecution of the gradient with the same slope as before t_4, and the dual slope elution. This analysis was conducted for different duration of the elution phase. To set a breakpoint in which the second gradient elution was introduced, the characteristic times (t_1 – t_5) were reinvestigated for each elution duration in the batch operation summarized in Table 1 by offline analysis of the fractions collected during each experiment. For each batch purification, by setting a purity specification (P_{spec}) as 80%, which was an arbitrarily chosen value for this purification, a maximum yield was obtained from an optimal product pool (t_3 – t_4 interval). Therefore, the most suitable breakpoint to introduce the second gradient elution was at t_4, in which all the recoverable product was collected at the purity ≥ P_{spec}. For the different elution modes, only the slope from t_4 to t_5 was changed in the form of either steeper (dual) or constant (isocratic); the gradient elution from t_1 was linearly increased until t_4, and then, in the dual slope elution, a sharp increase of the elution buffer was employed from t_4 to t_5, whereas in the isocratic elution, the concentration of the elution buffer was kept constant from t_4 to t_5. As an example, in the case of the 10 CV gradient elution, the elution duration corresponding to t_4 was 6 CV, which corresponded to 60% equilibration buffer and 40% elution buffer. Starting from the single gradient elution, the gradient slope was kept constant from 40% to 60% elution

Table 1
Batch operation using the three gradient elution modes for PEG-lysozyme purification.

Phase	Volume (CV)	Remarks	
Equilibration	3.00	Equilibration buffer(20 mM NaPi, pH 6.1)	
Load Feed	4.00	PEG-lysozyme (5 g L^{-1})	
Wash	3.00	Equilibration buffer	
Elution in the interval t_1-t_4	3.35[a], 6.00[b], 8.50[c]	Elution buffer (25 mM NaPi, 0.5 M NaCl, pH 6.1)10.0 – 40.0% linear gradient elution	
Elution in the interval t_4-t_5	**Isocratic** Elution buffer 40.0–40.0% Duration (CV) 6.00[a,b,c]	**Single** Elution buffer 40.0 – 60.0% Duration (CV) 1.65[a] 4.00[b] 6.50[c]	**Dual** Elution buffer 40.0 – 100.0% Duration (CV) 2.00[a,b,c]
Strip	2.00	Strip buffer (25 mM NaPi, 1.0 M NaCl, pH 6.1)	
CIP	2.00	CIP buffer (0.5 N NaOH)	
Re-Equilibration 1	1.00	Elution buffer	
Re-Equilibration 2	3.00	Equilibration buffer	

[a] Total elution duration (t_1-t_6)=5 CV.
[b] Total elution duration (t_1-t_6)=10 CV.
[c] Total elution duration (t_1-t_6)=15 CV.

buffer over 4 CV gradient elution, thereby operating at 10 CV elution duration and the same gradient slope. To introduce the dual slope elution, the second gradient elution was conducted from 40% to 100% elution buffer over 2 CV elution duration. To introduce the isocratic elution, the 40% elution buffer concentration reached at t_4 was kept constant for further 6 CV. The summary of this experimental design is shown in Table 1. All fractions in the three gradient elution modes were collected and analyzed with WCX-HPLC as discussed in 2.2.

As shown in the case of PEG-lysozyme for introducing the second elution gradient, a similar procedure was applied to the batch purification of PEG-target. First, the characteristic times t_1 – t_5 were identified by off-line analysis of the fractions collected during the batch experiments performed under the conditions reported in **Table S2**. Starting from the optimal design batch, which had been already investigated in a previous study, the characteristic time t_4 was identified [18]. The preparative batch purification was performed using a HiScreen Q FF column (7.7 × 100 mm, CV = 4.65 mL, particle size 90 μm) on the same ContiChrom CUBE 30. The process was conducted at a constant velocity 200 cm hr^{-1} (RT = 3 min) with its feed concentration of 1.05 g L^{-1}. Its composition is analyzed by the SEC–HPLC analytics. In the elution phase, by using 50 mM NaCl concentration in the elution buffer, the linear gradient elution started from 5 mM NaCl (*i.e.* 90% equilibration buffer/10% elution buffer) and ended at 50 mM NaCl concentration (*i.e.* 0% equilibration/100% elution buffer).

It should be pointed out that in this optimal design batch of PEG-target purification, the characteristic times t_4, t_5, and t_6 were equal, and thus the first elution duration was set to 10 CV, equal to the elution duration in the optimal design batch in **Table S2**. However, these process conditions led to 25% product loss in the strip phase, and neither increasing the elution duration nor increasing the modifier concentration of the elution buffer allowed to reduce this loss. Therefore, the introduction of a second elution phase for this specific product is very beneficial because it allows to recover this product to be recycled internally, namely introducing a discrete interval t_4 – t_5.

In Table 2, the batch operation methods using different modifier concentrations in the second elution phase are reported. A new elution buffer (20 mM Tris, 200 mM NaCl) was introduced to conduct these experiments, replacing the 50 mM NaCl elution buffer for the introduction of the dual slope. Due to the change in the modifier concentration, the elution buffer percentage was shifted accordingly. In the first elution phase, the 5 – 50 mM NaCl linear gradient elution was achieved by increasing the elution buffer volume percentage from 2.5% to 25%. Subsequently, to introduce and find an optimal dual slope, linear elution gradients starting at the modifier concentration at t_4, which was 50 mM NaCl, and ending at higher modifier concentrations (100, 125, 150, 175, and 200 mM NaCl) were investigated with two different elution durations (*i.e.* 2 and 3 CV). Fractions were collected during the second elution duration and analyzed by the SEC analytics.

2.4. MCSGP operation

Using the buffers and operating parameters obtained from the batch experiments, MCSGP operation was designed. In particular, the characteristic times determined for the W/P (*i.e.* t_2 – t_3 interval) and P/S (*i.e.* t_4 – t_5 interval) regions were used to define the product-containing side fractions to be internally recycled after an appropriate inline dilution by the equilibration buffer.

An additional operating parameter is refeed phase from the second switch, due to a partial feed that is introduced by the recycled overlaps. In the start-up of MCSGP, the upstream column undergoes the same equilibration and load feed phases as the design batch does, but from the second load feed, a partial feed is already loaded onto the downstream column. If the same fresh feed amount as the upstream column was loaded onto the downstream column, the load density on this downstream column would exceed the target load density. To avoid overloading, the mass of the recycled overlaps is subtracted from the feed to compute the second load feed amount.

After the end of the start-up, the upstream column proceeds to the wash and elution phases and the downstream column from the equilibration and (second) load feed as shown in Table 3 for PEG-lysozyme and in Table 4 for PEG-target.

In the PEG-lysozyme MCSGP operation, the elution duration of 10 CV was selected for the investigation of the three gradient elution modes, which were single gradient, dual slope, and isocratic elution. As discussed in Table 1, the breakpoint from the first to the second elution phases was determined as the characteristic time t_4, corresponding to 6 CV in this experimental setting. While the MCSGP operation for the single gradient elution kept its gradi-

Table 2
Batch operations using dual slope for PEG-target purification.

Phase	CV	Remarks				
Equilibration	5.00	Equilibration buffer (20 mM Tris, pH 7.8 – 8.2)				
Load Feed	3.81	PEG-target (1.05 g L^{-1})				
Washing	5.00	Equilibration buffer				
Elution in the range t_1-t_4	10.00	Elution buffer (20 mM Tris, 200 mM NaCl, pH 7.8 – 8.2) 2.5% - 25.0% linear gradient elution				
Elution in the interval t_4-t_5	2.00, 3.00	100 mM 25.0% - 50.0% linear gradient elution	125 mM 25.0% - 62.5% linear gradient elution	150 mM 25.0% - 75.0% linear gradient elution	175 mM 25.0% - 87.5% linear gradient elution	200 mM 25.0% - 100.0% linear gradient elution
Strip	3.00	Strip buffer (1 M NaCl)				
Sanitization	5.00	CIP buffer (1 M NaOH)				
Storage	3.00	Storage (20% EtOH)				

Table 3
MCSGP for PEG-lysozyme.

Phase	Volume (CV)	Superficial velocity (cm hr^{-1})	Remarks
Equilibration	3.00	300.0	Equilibration buffer
Load from W/P recycle	1.00^{a,b,c}	Inline dilution = 204.5^{a,b,c} W/*P* = 95.5^{a,b,c}	PEG-lysozyme (5.00 g/L) W/P: weak impurity-product recycles come from the elution phase of the second column
Fresh Feed Load	3.45^{a}, 3.38^{b}, 3.39^{c}	300.0	
Load from P/S recycle	1.00^{a}, 0.65^{b}, 2.35^{c}	Inline dilution = 231.9^{a}, 249.8^{b}, 224.8^{c} P/*S* = 68.1^{a}, 50.2^{b}, 75.2^{c}	P/S: Product-strong impurity recycles come from the elution phase of the second column
Wash	3.00	300.0	Equilibration buffer
Elution 1 (from t_1 to t_4)	6.00^{a,b,c}	W/P recycle – 95.5^{a,b,c} Product collect – 124.2^{a,b,c}	Elution buffer 10.0 – 40.0%^{a,b,c} linear gradient elution
Elution 2 (from t_4 to t_5)	4.00^{a}, 2.00^{b}, 6.00^{c}	P/S recycle – 68.1^{a}, 50.2^{b}, 75.2^{c}	Elution buffer Single: 40.0 – 60.0% linear gradient elution Dual: 40.0 – 100.0% linear gradient elution Isocratic: constant at 40%
Strip	2.00	300.0	Strip buffer
CIP	2.00	300.0	CIP buffer
Re-equilibration 1	1.00	300.0	Equilibration buffer
Re-equilibration 2	3.00	300.0	Equilibration buffer

[a] Single gradient elution.
[b] Dual slope elution.
[c] Isocratic elution.

ent elution constant with its duration of additional 4 CV, the isocratic elution with its duration of additional 6 CV or the dual slope elution with additional 2 CV were conducted in the second elution phase between t_4 and t_5, which was also the P/S recycling fraction in this operation. The MCSGP was operated for 4 cycles, or 9 switches, for all three gradient elution modes. Fractions were collected for each switch and analyzed by the WCX analytics. Yield and productivity were calculated using Eqs. (5) and (6), respectively, where the load amount of each switch ($t_{\text{load per switch}}$) was addressed. The first switch load was equal to the load in the design batch, which was 8.0 min (*i.e.* 4.0 CV), and the subsequent switch load was as reported in Table 3.

$$Yield\ (\%) = \frac{m_{recovered\ \ per\ \ switch}}{m_{total\ \ per\ \ switch}} \times 100\% \tag{5}$$

$$Productivity = \frac{m_{recovered\ \ per\ \ switch}}{CV \times t_{total\ \ per\ \ switch}} = \frac{C_{feed} \times Q_{feed} \times t_{Load\ \ per\ \ switch}}{CV \times t_{total\ \ per\ \ switch}} \times Yield \tag{6}$$

A further investigation on the dual slope elution was conducted in the MCSGP operation of PEG-target. The 125 mM NaCl elution buffer was selected as a representative case study to evaluate the feasibility of introducing a P/S recycling fraction in this particular separation. As already mentioned previously, the characteristic time t_4 was at 10 CV, the dual slope with its durations (2 and 3 CV) was introduced after this breakpoint. The operation was conducted for 4 cycles, or 9 switches, and fractions of each switch were collected and analyzed with the SEC analytics. Using the same equations Eqs. (5), and (6), yield and productivity were computed.

3. Results and discussion

3.1. Breakpoint definition via batch chromatography

With this systematic study, we want to investigate the role of the gradient slope in the P/S recycling region (*i.e.* $t_4 - t_5$) on the performances of MCSGP deputed to isolate the mono-PEGylated compound. Two case studies were analyzed, namely the purification of PEG-lysozyme and of the industrial protein PEG-target. The analytical chromatograms of the crude mixture to be purified are reported in Figure S1.

For this purpose, the first task was the proper identification of the 6 breakpoints $t_1 - t_6$ under different elution durations. This was done from the single-column batch purification of the two PEGylated proteins. 50 fractions were collected in the elution phase

Table 4
MCSGP for PEG-target.

Phase	Volume (CV)	Superficial velocity (cm hr^{-1})	Remarks
Equilibration	5.00	200.0	Equilibration buffer
Load from W/P recycle	1.30[a,b]	Inline dilution = 93.0[a,b] W/P = 107.0[a,b]	PEG-target (1.05 g L^{-1}) W/P: weak impurity-product recycles come from the elution phase of the second column
Fresh Feed Load	2.78[a,b]	200.0	
Load from P/S recycle	3.00[a], 2.00[b]	Inline dilution = 184.1[a], 181.7[b] P/S = 15.9[a], 18.3[b]	P/S: Product-strong impurity recycles come from the elution phase of the second column
Wash	5.00	200.0	Equilibration buffer
Elution 1 (from t$_1$ to t$_4$)	10.00[a,b]	W/P recycle - 107.0[a,b] Product collect – 156.0[a, b]	Elution buffer Start: 4% to 40% linear gradient elution
Elution 2 (from t$_4$ to t$_5$)	3.00[a], 2.00[b]	P/S recycle – 15.9[a], 18.3[b]	Elution buffer End: 40% to 100% linear gradient elution
Strip	3.00	200.0	Strip buffer
Sanitization	5.00	200.0	CIP buffer
Storage	3.00	200.0	Storage buffer

[a] 50 mM + 125 mM 3CV dual slope elution.
[b] 50 mM + 125 mM 2CV dual slope elution.

and analyzed through HPLC in order to track the concentration of the product and the different impurities during the elution time.

By knowing this profile and by defining a certain purity specification (P$_{spec}$) for the two molecules as discussed in the following, it is possible to identify, at each elution time (t$_{elu}$) investigated, the product collection window (*i.e.* t$_3$ – t$_4$) allowing to satisfy this constraint on the purity while maximizing the yield [5,18]. After the optimal pair of t$_3$ and t$_4$ that gives the highest yield with the satisfactory purity is identified, its process performance is further evaluated by computing the productivity. Finally, the W/P and the P/S recycling regions could be determined as the intervals from t$_2$, when the product starts to elute, to t$_3$ and from t$_4$ to t$_5$, by evaluating when the product ends to elute, respectively. In the following, the discussion on the definition of these breakpoints and the batch process performances at different t$_{elu}$ and gradient slope between t$_4$ and t$_5$ are specifically discussed for the two molecules under examination.

3.1.1. PEG-Lysozyme

For PEG-lysozyme we set a minimum acceptable purity P$_{spec}$ to 80%. In order to study the impact of the second elution phase and identify the breakpoint (t$_4$) where it could be introduced, batch purifications using three different elution durations were performed. As shown in Figure S2, the breakpoint t$_4$ for 5, 10, and 15 CV elution durations were 29.2, 34.5, and 39.5 min, respectively. The corresponding elution durations until t$_4$ were 3.35, 6.00, and 8.50 CV, respectively. This means that in the case of the 10 CV elution duration, as an example, the highest yield satisfying P$_{spec}$ was reached at 6.0 CV elution duration, which corresponded to 40% of the elution buffer.

Having defined these breakpoints for the three t$_{elu}$, an earlier stop of the first elution phase was introduced. From t$_4$ onward, the batch purifications were performed with the introduction of the second elution phase. In the case of a total elution of 10 CV, this second phase was either: i) an isocratic elution at 40% elution buffer for 6 CV, ii) a continuation of the elution phase with the same gradient slope for further 4 CV until 60% of elution buffer or iii) a steeper gradient slope up to 100% elution buffer in 2 CV. The resulting chromatograms are overlaid and shown in Fig. 2. The fractions collected during the two elution phases were analyzed via WCX-HPLC, and the relative abundance of the different species are shown in Figure S3. As evident from the superimposed chromatograms, a good overlay until t$_4$ was worth highlighting. Theoretically, as no parameter was changed from the beginning of these purifications to t$_4$, we expected the same W/P region (*i.e.* t$_2$ – t$_3$) and the optimal collection window (*i.e.* t$_3$ – t$_4$) resulting in the same yield at the purity ≥ P$_{spec}$.

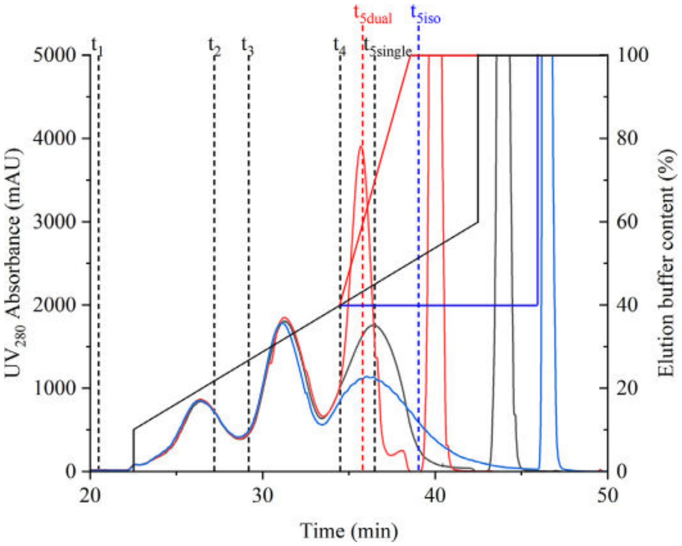

Fig. 2. Superimposed chromatograms of the three different elution methods. The UV absorbance at 280 nm and elution buffer concentration at the outlet of the column of the single (black), dual slope (red), isocratic elution (blue) are shown for the exemplary process with a total elution duration of 10 CV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The theoretical assumption of the same optimal pair was confirmed by our analytics. All performance parameters and critical characteristic times are summarized in Table S3. Despite some experimental deviations were observed in the analytics in terms of purity and yield, the purity specification was satisfied, and the yield difference within the same elution duration was not significant. The trend of using different elution durations was evident from the yield values: increased elution durations led to higher resolution of the separation and hence higher yields at the imposed P$_{spec}$. The next most notable difference in this table was the difference in t$_5$ within the same elution duration. Across all elution modes, the dual slope elution showed the shortest t$_4$ – t$_5$ interval and the isocratic the longest interval. Due to the difference in the t$_5$, the productivity for the dual gradient elution was actually improved for this batch process as the total time of the purification decreased while the purity and yield were assured to be equivalent to those of the single gradient elution. On the other hand, the

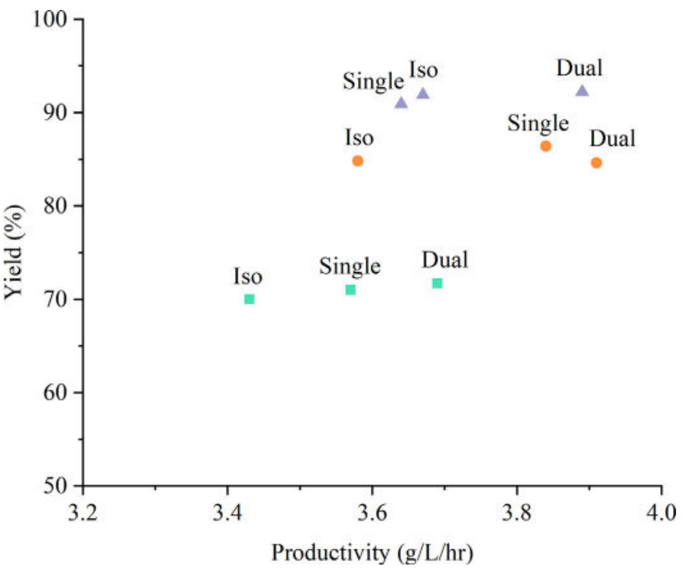

Fig. 3. Summary of performance parameters of PEG-lysozyme batch purifications for processes with total elution duration of 5 CV (■), 10 CV (●), and 15 CV (▲) in the three configurations for the second elution phase: isocratic, single slope, dual slope.

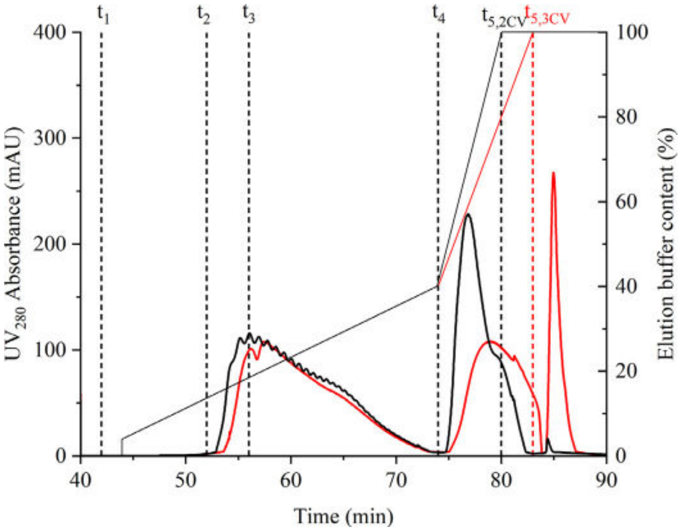

Fig. 4. Superimposed chromatograms of different second elution phase durations using 125 mM NaCl. The UV absorbance at 280 nm and elution buffer concentration at the outlet of the column are represented for 2 CV (black) and 3 CV (red) second elution durations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased t_5 by the isocratic elution generally decreased the productivity relative to the single gradient elution, with an exception of the 15 CV elution duration, where the single gradient elution was already sufficiently gradual in its elution and thus sufficient separation was achieved, resulting in the same t_5 as the isocratic elution.

Overall, as expected, the introduction of a steeper elution after t_4 allows preserving high yield and increases the productivity compared to the other modalities, as evident from Fig. 3. Therefore, for a single-column batch process, this dual elution mode is undoubtedly beneficial.

3.1.2. PEG-target

A similar analysis was then applied to the industrially relevant PEG-target purification. From our previous study, we demonstrated that this molecule is very peculiar as a part of the product could not be collected in the elution phase but rather is lost in the strip phase [18]. In addition, the optimal collection window is protracted until the beginning of the strip, with t_4 coinciding with t_5 (see Figure S4). This makes the analysis of the single gradient and isocratic elution meaningless for this molecule. Conversely, a second elution phase with a steeper gradient was introduced in this case to improve the product recovery and to generate a net $t_4 - t_5$ interval to be used as recycling region in the MCSGP.

To evaluate the effect of this modification, a 10 CV elution duration using 50 mM NaCl elution buffer was applied until t_4. After this point, a second elution phase with a steeper gradient was applied in the new interval $t_4 - t_5$. Two durations of 2 and 3 CV for this phase were investigated, each at different gradient boundaries. Superimposed chromatograms when using 125 mM NaCl as the upper gradient boundary for the 2 and 3 CV elution durations are shown as an example in Fig. 4.

It is possible to observe that the two chromatograms match well until t_4, where the first elution phase is the same for both. On the other hand, the application of a second elution phase after t_4 allows to further separate the residual product that was lost in the strip during a single elution (Figure S4) from the strong impurities. This is important to generate a P/S recycle for the MCSGP and increase the product recovery.

From the batch experiments conducted at varying slopes of the second elution phase and upper gradient boundaries, this potential gain in the product yield could be accounted for by evaluating the relative abundance of product pushed in the second elution phase, as shown in Figure S5 and summarized in Table S4. As expected, having higher modifier concentrations and longer elution durations allowed for higher potential yield gains. By using the 200 mM NaCl buffer for the 3 CV elution duration, the maximum yield of 99.3% was achieved in the hypothetical MCSGP operations.

Again, as long as single-column operations are involved, a second elution phase proved to be beneficial to the process.

The convenience in implementing this new elution mode is discussed in the following for the MCSGP, where the beneficial effect of compressing the P/S recycling volume is contrasted by the higher dilution factor required.

3.2. MCSGP

After having defined the breakpoints and investigated how the different gradient slopes in the interval $t_4 - t_5$ affect the performances of a single-column batch purification, we moved to the design of MCSGP, where the P/S recycling comes into play in that specific time interval. The two case studies of PEGylated lysozyme and PEG-target are discussed individually in the following.

3.2.1. PEG-Lysozyme

For the implementation of MCSGP, the 10 CV elution duration case was selected. The operating parameters (*i.e.* load amount, elution duration, and characteristic times) as determined in the batch process were directly transferred to the design of the MCSGP operation as illustrated in Figure S6. As discussed before, t_2, t_3 and t_4 (27.2, 29.2, and 34.5 min) were set equal in all three elution modes (*i.e.* single slope, dual slope and isocratic elution), while t_5 is a function of the second gradient elution.

Four cycles including the start-up and shut-down phases were performed for each of the three elution modes. As illustrated in Fig. 5, the good overlapping of the chromatograms collected during the different cycles confirmed that a steady state was reached after the 2nd cycle in this continuous operation.

The summary of the performance parameters and process gains across the different elution modes are shown in Table S5 and visu-

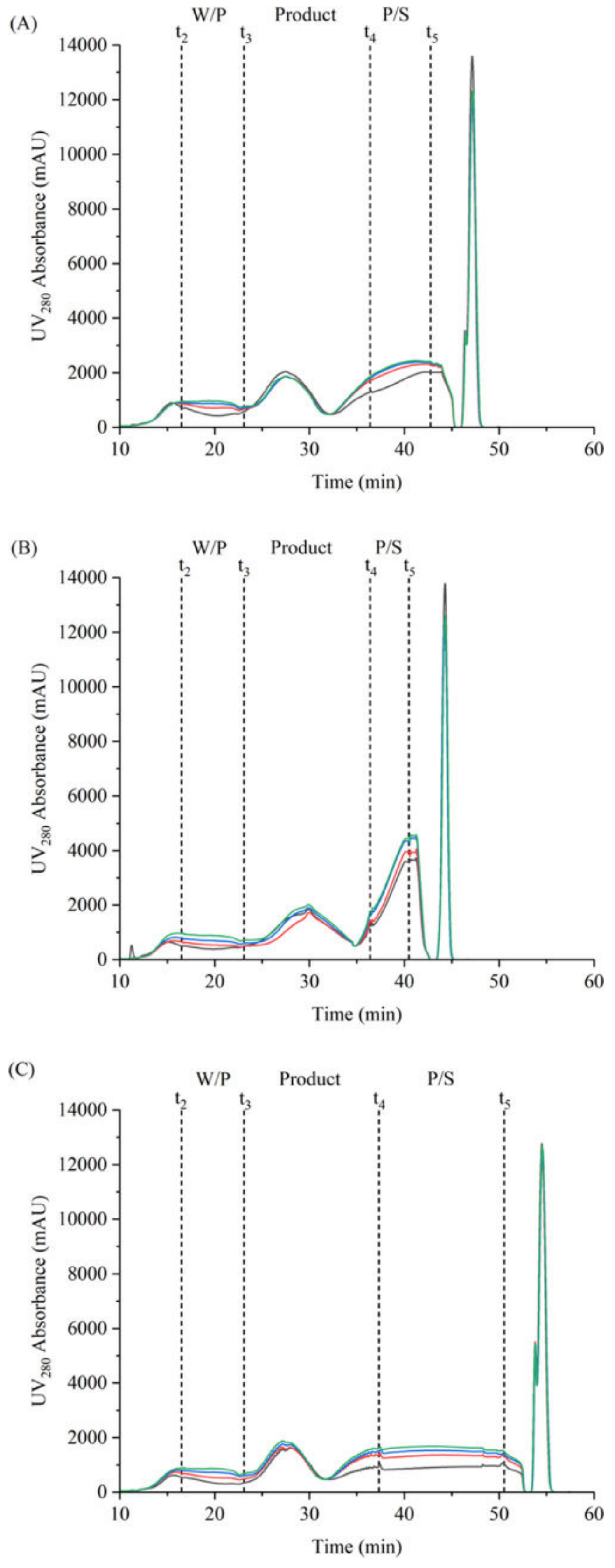

Fig. 5. Superimposed MCSGP chromatograms of (A) Single slope, (B) Dual slope, and (C) Isocratic elution. The UV absorbance at 280 nm at the outlet of the first column during the first (black), second (red), third (blue), and fourth (green) cycle, and dotted vertical lines for the characteristic times t_2 – t_5 are represented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

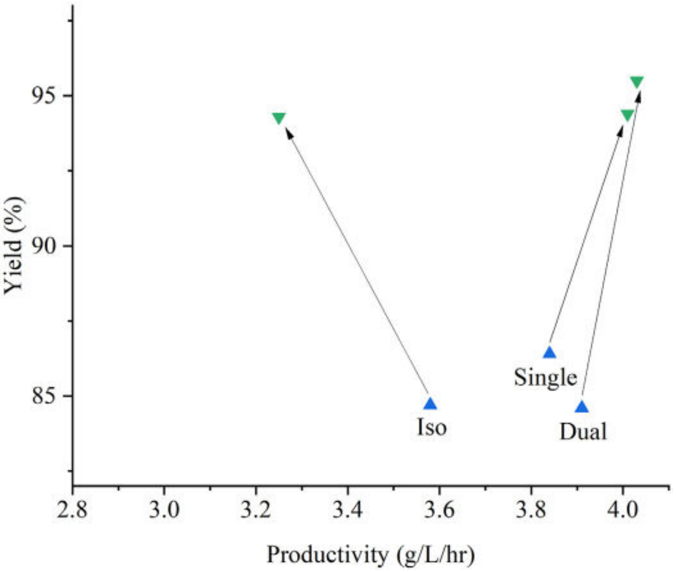

Fig. 6. Performance parameter comparisons moving from batch to MCSGP for the three gradient elution modes. Batch (▲) and MCSGP (▼) with its gradient elution mode labeled are represented.

alized in Fig. 6. As observed in the superimposed chromatograms, starting from the third cycle, the MCSGP operations were in the steady state. Therefore, the last four switches were used for computing the average performance parameters of MCSGP, which were then compared with those of the batch process. From Fig. 6 it is possible to observe that MCSGP accesses higher product yield (+10%) compared to the corresponding batch, regardless of the elution mode, with comparable purity above P_{spec}.

Coming to the comparison of the different elution modes, the MCSGP operated with the dual slope was able to maximize both yield and productivity. The latter in particular can be explained with a compression in the volume of the P/S recycling region, driven by the steeper gradient in the second elution. The higher dilution factor and the consequent decrease in the flow rate for the recycle of the P/S overlap does not seem to erode completely this shortened recycling duration, which then drives the performance of the MCSGP operation. At the same time, a steep increase in the modifier concentration allowed to gather most of the product in this side-fraction, to be recycled to the downstream column for further processing. On the other hand, the isocratic MCSGP operation showed the lowest productivity, even lower than its batch counterpart (3.58 to 3.25 g L^{-1} hr^{-1}). This decrease was attributed to an elongated P/S recycle time as t_5 was the highest among the three different elution modes despite having the lowest inline dilution factor. This actually indicated that the mild gradient elution with the increased t_4 – t_5 interval was not suitable for this particular separation, thereby increasing the recycling time in the P/S interval. Overall, for this peculiar separation, a second and steeper gradient in the interval t_4 – t_5 is beneficial in improving the performances of MCSGP, despite requiring the highest dilution factor to allow the binding of the recycled fraction in the downstream column. It is then a valuable option for boosting this process and make it appealing from a scale-up point of view.

3.2.2. PEG-target

The convenience of introducing a dual gradient slope in MCSGP of the industrial protein PEG-target was finally evaluated. In a previous work, the MCSGP operation conducted with 10 CV elution duration, 50 mM NaCl elution buffer, and with a single gradient slope already showed process gains compared to the batch process

[18]. From that study, the optimal characteristic times from t_2 to t_4, which were 52.0, 56.0, and 74.0 min, were applied to the design of the dual slope elution MCSGP as shown in Figure S7. Furthermore, the steeper gradient introduced in the second elution phase allowed to further separate the residual product lost in the strip in the traditional process, as already demonstrated during the batch experiments (see Fig. 4). This allowed to define a P/S recycling window from t_4 to t_5, with the latter equal to 80.0 min and 83.0 min for the 2 CV and 3 CV elution durations, respectively. It is worth highlighting that, while these times apply to the batch process used to design the MCSGP, the product collection interval in the actual MCSGP was expected to extend due to the decreased flow rate from 200 cm hr^{-1} to 156 cm hr^{-1} to process the same volume, in order to ensure the same residence time in the receiving column when inline dilution is added. This changes the breakpoints proportionally, as is illustrated in Fig. 7**A**, in which the product collection interval of the MCSGP operation is now 23 min instead of 18 min. The same process was also applied for the W/P and P/S recycling fractions considering the different recycling flow rates reported in Table 4. On the other hand, the application of the single and the isocratic elution modes was not considered in this section as they do not allow the definition of this P/S recycling window and hence do not bring new information compared to what reported in [18].

For the MCSGP operations, the 125 mM NaCl elution cases for both 2 CV and 3 CV elution durations were investigated. After running 4 cycles, each fraction was collected and analyzed. The summary of the performance parameters is shown in Table S6. As shown in Fig. 7**A** and **B**, the steady states for the two durations were reached at the third cycle. Therefore, the performance parameters of the last four switches were averaged to draw a comparison between the batch and MCSGP. In this latter case, for the 2 CV second elution duration, the yield and productivity were 93.0% and 0.60 g L^{-1} hr^{-1}, and for the 3 CV second elution duration, 95.9% and 0.48 g L^{-1} hr^{-1}, respectively. The general observation was a marked improvement in the product yield compared to the batch process. The introduction of a second elution phase and the possibility of recycling the P/S overlap in the MCSGP allowed to recover most of the product (>93%) discarded within the strip in the case of the batch process (maximum yield = 73%). This gain is extremely important for high-value products but also for difficult-to-produce molecules, as such a higher recovery in the downstream section reduces the pressure on the upstream processing when a required production output needs to be met. Unfortunately, this improvement comes at the cost of a reduced productivity, affected by the high dilution factor required for this P/S recycling and hence the decisive reduction in its flow rate when the downstream column needs to be loaded at constant velocity.

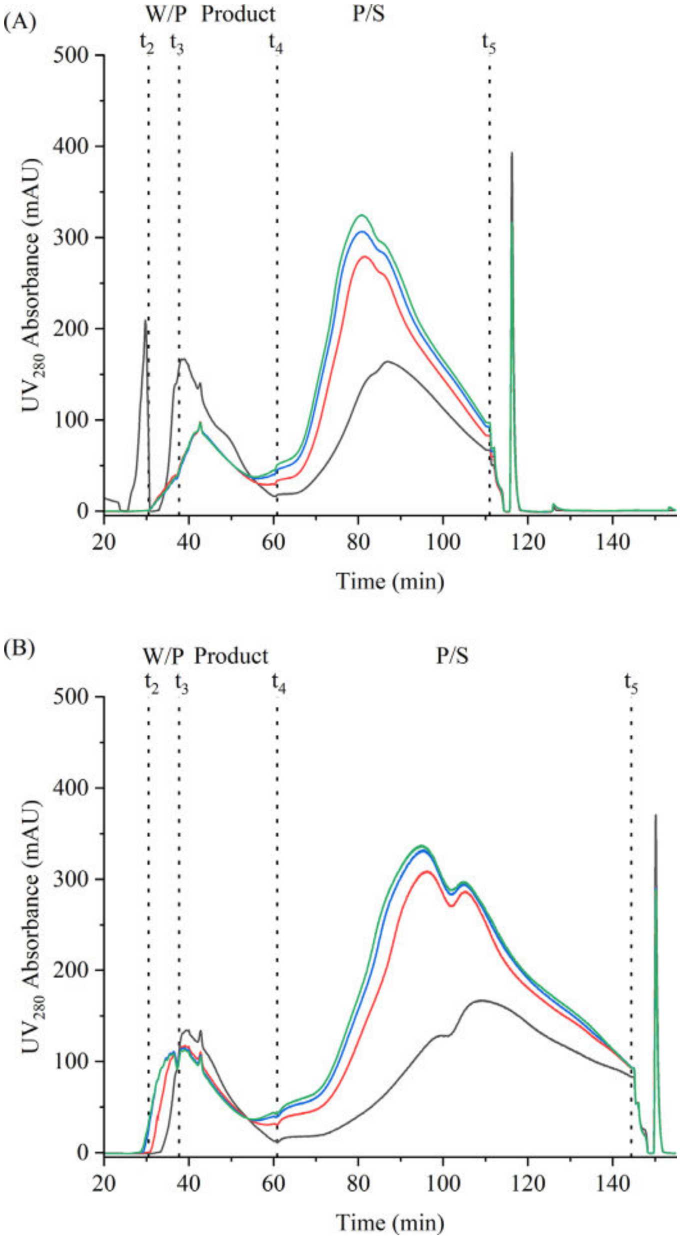

Fig. 7. Superimposed MCSGP chromatograms of (A) 2 CV and (B) 3 CV. The UV absorbance at 280 nm at the outlet of the first column during the first (black), second (red), third (blue), fourth (green) cycle, and dotted vertical lines for the corresponding collection interval are represented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To better highlight this tradeoff between yield and productivity, MCSGP operations at different NaCl upper boundary were simulated starting from the batch experiments. The results are illustrated in Fig. 8, where close proximities between hypothetical and experimental performances are observed. While the higher modifier concentrations offered the higher expected yield gains, increased inline dilution factors leading to higher switch time was not favorable in terms of productivity. In the case of the 3 CV elution duration, for example, using 100 mM NaCl elution buffer required 12.04-fold inline dilution that led to a cycle lasting 4.80 h. On the other hand, using 200 mM NaCl elution buffer required a dilution factor of 15.21. This extended the duration of a single cycle to 5.80 h. As the process until t_4 was kept the same, the only factor that impacted the cycle duration was indeed the inline dilution factor. This variation due to changes in the inline dilution factor evidently impacts the productivity. In particular, despite an increase in the modifier concentration led to a compression in the P/S volume to be recycled, the reduced flow rate necessary to dilute this fraction led to a larger process duration. At the same time, the higher potential yield gain of 24.3% could be achieved by using 200 mM NaCl elution buffer as the high modifier concentration allowed PEG-target and the strong impurity to be eluted in the elution phase instead of wasting them into the strip, while using 100 mM elution buffer still lost a significant amount of the PEG-target due to its lower modifier concentration. Consequently, increasing the modifier concentration of the elution buffer could increase the yield but at the expense of increasing the cycle time, which decreased the overall productivity. This led to a Pareto front in which high yields were achieved at the cost of the productivities and *vice versa*. From this plot, the highest hypothetical productivity was obtained using 2 CV elution duration and the 100 mM NaCl elution buffer. The shortest cycle time (3.68 h) out-compensated the decrease of the hypothetical yield of 90.5% to give the high-

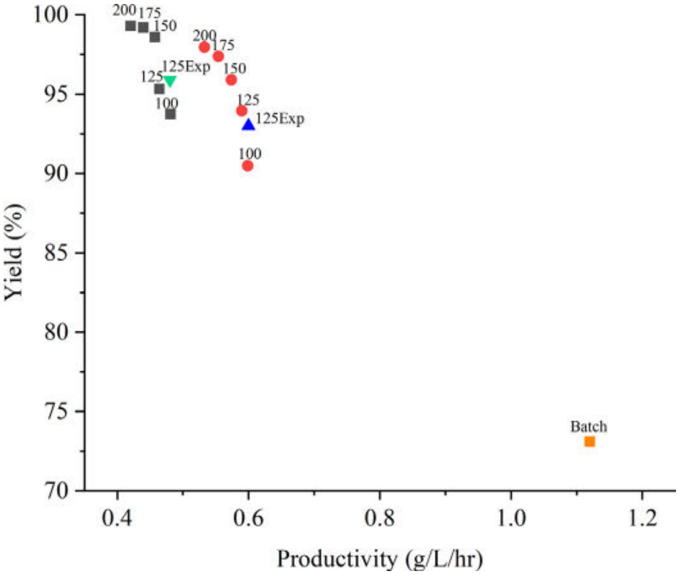

Fig. 8. Performance parameters for a dual slope MCSGP of PEG-target at different NaCl concentrations and comparison with the hypothetical 2 CV (■) and 3 CV (•) and experimental 2 CV (▼) and 3 CV (▲) elution durations using the 125 mM NaCl elution buffer and with the optimal batch experiment (■). The label on each point indicates the elution buffer salt concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

est productivity of 0.60 g L^{-1} hr^{-1}. The highest yield was obtained using the opposite end of these experimental conditions, the 3 CV elution duration with the 200 mM NaCl elution buffer. The hypothetical yield in this case was 99.3% but with the lowest productivity of 0.42 g L^{-1} hr^{-1}. The lowest productivity was also attributed to the extended cycle time (5.80 h), which was increased by two factors: i) the high inline dilution factors (15.21-fold dilution) and ii) the longer elution duration compared to the 2 CV elution duration.

4. Conclusions

In this work, we investigated the role of the gradient slope during the internal recycling of the product-containing side-fraction P/S in MCSGP of PEGylated proteins, as this affects the yield and productivity of the process.

The general procedure was illustrated in the ideal purification of PEG-lysozyme, where identical process parameters were set until t_4 and then three possibilities for the gradient slope, namely isocratic, same slope and dual slope were thoroughly investigated in the t_4 – t_5 interval. As all operating parameters until t_4 were the same, comparable purities and yields were observed for the three situations. At the same time, the dual slope allowed to reduce the overall process duration, leading to an improvement in the productivity. Then, for this molecule, the compression of the volume to be recycled impacts the process duration more than the higher in-line dilution factor, responsible for a reduction in the elution flow rate.

Given the advantages of the dual slope elution shown in the PEG-lysozyme case, this elution mode was further studied in the PEG-target purification. Here, the increase in the modifier concentration by the dual slope allowed to push the product originally discarded with the strip in the elution phase, and then to generate a P/S recycling window in MCSGP. This was extremely beneficial in terms of product recovery, which was pushed up to 99.3%. However, this marked improvement in the yield (+27%) came at the cost of sacrificing the productivity, which for the 2 CV elution duration was 0.60 g L^{-1} hr^{-1}, while for the corresponding batch it was 1.21 g L^{-1} hr^{-1}. Considering the previous work of PEG-target purification that reached 79.1% yield and 1.32 g L^{-1} hr^{-1} productivity with the single-gradient MCSGP operation [17], this significant yield improvement can potentially alleviate demand on upstream bioprocessing capacity as less product is wasted. At the same time, available processing time needs to be considered in implementing this dual slope mode as decrease in productivity will be observed.

Therefore, this strategy of introducing a dual gradient is particularly advantageous when applied to MCSGP of high-value and difficult-to-produce molecules. However, a careful investigation on the operating ranges must be conducted as this rapid change in modifier concentrations may shift the thermodynamics of the separation [40,41]. This approach might not be universal but could certainly offer an alternative to proteins with long tailing peaks, which overlap with impurities, by using the countercurrent chromatography. As we encounter many difficult-to-separate products in this downstream processing field, with the combination of the batch optimization strategy, this new technique of MCSGP operation offers a solution to reach near 100% yield, which allows to increase the revenues but also to reduce the pressure on the upstream manufacturing when a certain product demand needs to be met.

Credit author statement

All authors have read and given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interests.

CRediT authorship contribution statement

Tae Keun Kim: Investigation, Data curation, Writing – original draft. **Abdallah Ayub Bham:** Validation. **Ismaele Fioretti:** Validation. **James Angelo:** Conceptualization, Writing – review & editing, Funding acquisition. **Xuankuo Xu:** Conceptualization, Writing – review & editing, Funding acquisition. **Sanchayita Ghose:** Funding acquisition. **Massimo Morbidelli:** Funding acquisition, Supervision, Writing – review & editing. **Mattia Sponchioni:** Resources, Supervision, Project administration, Writing – review & editing.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.463868.

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